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Monitoring genetically modified rhizobia in field soils using the polymerase chain reaction

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D.W. CULLEN, P.S. NICHOLSON, T.A. MENDUM AND P.R. HIRSCH. 1998. Monitoring genetically modified (GM) bacterial inoculants after field release using conventional culture methods can be difficult. An alternative is the detection of marker genes in DNA extracted directly from soil, using specific oligonucleotide primers with the polymerase chain reaction (PCR). The PCR was used to monitor survival of two GM *Rhizobium leguminosarum* bv. *viciae* inoculants after release in the field at Rothamsted. One strain, RSM2004, is marked by insertion of transposon Tn*5*; the second strain, CT0370, released at the same site, is modified by chromosomal integration of a single copy of the gene from *E. coli* conferring GUS activity. Both GM strains provide a realistic case study for the development of PCR-based detection techniques. Specific primers were developed to amplify regions of the Tn*5* and GUS genetic markers using PCR and conditions optimized for each primer set to routinely detect a signal from 10 fg of purified template DNA, the equivalent of one cell per reaction. Procedures to improve the sensitivity of detection are described, to detect fewer than 50 cells g^{-1} soil in soil-extracted DNA.

INTRODUCTION

Monitoring the survival and spread of microbial inoculants after addition to field soils is important for assessing their efficacy; it is essential when GM bacteria are released. Detection using conventional culture methods may not be possible when numbers are low relative to the indigenous population and no selective media are available. However, it is possible to detect specific DNA sequences in soil microbial populations by the extraction of total microbial DNA from environmental samples, combined with DNA probes and PCR. This approach enables sensitive detection of any group of bacteria for which probes are available, including those in non-culturable states. The extraction of DNA from soils and the application of DNA probes and PCR amplification has been reported previously (Tsai and Olson 1991; Pillai *et al.* 1991; Selenska and Klingmüller 1992; Smalla *et al.* 1993; Flemming *et al.* 1994). Previous studies on soil DNA extraction and PCR sensitivity have involved 'reconstruction' experiments, adding organisms with marker sequences to soil and sediment samples prior to the extraction of total DNA after a relatively short incubation period (Steffan and Atlas 1988; Picard *et al.* 1992; Tsai and Olson 1992; Erb and Wagner-Döbler 1993; Tebbe and Vahjen 1993; Smalla *et al.* 1993; Flemming *et al.* 1994; Knaebel and Crawford 1995). These experiments do not provide realistic models for the development of PCR-based monitoring of GM bacteria because the behaviour and physical location of added indicator micro-organisms is unlikely to be the same as the indigenous population which will be adsorbed to clays, other minerals, and organic matter in a specific ecological niche, and thus potentially more difficult to lyse.

A simple, rapid and reliable method has been developed for extracting DNA directly from soils (Cullen and Hirsch 1997) and has been applied for monitoring GM rhizobia. Soil-extracted Tn*5* and GUS marker sequences from established field populations of two strains of *Rhizobium leguminosarum* bv. *viciae* could be reproducibly detected by PCR. The strains released at Rothamsted were designed to facilitate monitoring. RSM2004 (marked by insertion of the transposon Tn*5* on a symbiotic plasmid), released in 1987 is established at 10^2–10^3 culturable cells g^{-1} soil (Hirsch and Spokes 1994); CT0370 (marked by chromosomal insertion of the GUS gene from *Escherichia coli*, Selbitschka *et al.* 1995), released at the

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same site in 1994, is established at 10^4–10^5 culturable cells g^{-1} soil (Hirsch 1996). Thus, the field site provided an ideal opportunity to develop sensitive DNA-based methods for detection of GM rhizobia.

MATERIALS AND METHODS

Bacterial strains and release experiments

Parental strain RSM2001 has spontaneous resistance markers for rifampicin and streptomycin on the chromosome; its derivative RSM2004 has a single copy of Tn*5* (conferring neomycin/kanamycin resistance, used here as a selective marker, and also resistance to streptomycin and bleomycin) inserted on the single conjugative symbiotic plasmid (Hirsch and Spokes 1994). There is one copy of the Tn*5*-marked plasmid per cell, i.e. per RSM2004 genome. Strain CT0370 (Selbitschka *et al.* 1995) has a single copy of the *E. coli* GUS gene (*uid*A or *gus*A) inserted between the *rec*A and *ala*S gene in the chromosome, expressed from the neomycin phosphotransferase II promoter of Tn*5* (P_{npt}II) and spontaneous resistance markers for streptomycin and spectinomycin also located on the chromosome. The details of the rhizobial strains, release experiments, and methods for their enumeration on selective media, have been reported elsewhere (Hirsch and Spokes 1994; Selbitschka *et al.* 1995; Hirsch 1996). Rhizobia were cultured on Tryptone-Yeast agar (Hirsch and Skinner 1992) at 28 °C. Selective agar for plate counts to measure cfu (colony-forming units) was supplemented with appropriate antibiotics: for RSM2004, rifampicin and neomycin both at 100 μg ml^{-1}; for CT0370, streptomycin (500 μg ml^{-1}) and spectinomycin (200 μg ml^{-1}). When soil dilutions were plated, benomyl (7·5 μg ml^{-1}) and cycloheximide (100 μg ml^{-1}) were included to inhibit fungi. Duplicate or quadruplicate plate counts of soil dilutions were used to calculate cfu g^{-1} soil. There was a reduced plating efficiency of RSM2004 on neomycin (70%) and rifampicin (65%), the combined effect giving only 55% of the cfu on non-selective agar.

The plasmid pXS5, described here for the first time, consists of the 5·24 kb *HpaI* fragment of Tn*5* (Jorgensen *et al.* 1979) cloned into the *SmaI* site of pUC9 (Vieira and Messing 1982). This Tn*5* derivative, which lacks the terminal 180 bp of IS*50*L and IS*50*R (the Tn5 terminal repeats), is on a high copy number plasmid that can be propagated stably in *E. coli* to provide a convenient source of Tn*5* DNA.

DNA extraction from soil

The soil at the release site, the Rothamsted 'Garden Plots', is a silty clay loam (25% clay, 62% silt, 13% sand, pH 7·5). At each sampling time, 10 separate samples of about 50 g were taken from the field, mixed, and sieved (2 mm mesh) to provide sub-samples for DNA extraction or enumeration by plate culture. This was done as quickly as possible, at room temperature; usually, samples were processed within 30 min of collection from the field. Microbial DNA from soil was extracted using mechanical lysis, bead-beating 1 g soil with 1 g glass beads (equal weights of 0·5 mm, 2·0 mm and 3·0 mm diameter) in 3 ml 0·12 mol l^{-1} sodium phosphate buffer pH 8·0, 1% SDS for 5 min in a Mikro-dismembrator II (B. Braun, Melsungen, Germany) at amplitude 5 mm, followed by precipitation of organic contaminants with 5 mol l^{-1} potassium acetate pH 5·0 on ice, isopropanol precipitation of DNA from the supernatant fluid, and subsequent purification through polyvinylpolypyrrolidone (PVPP; Sigma) and Sephadex G75 spin-columns. Purity and concentration was assessed using spectrophotometry, fluorometry and agarose gel electrophoresis. After purification, DNA yields of 11·5 $\pm$ 2·7 μg DNA g^{-1} soil were obtained (mean, S.D. of 10 extractions). Details of all these methods are reported elsewhere (Cullen and Hirsch 1997).

To test the detection limit of the PCR for Tn*5*-marked RSM2004 in soil, DNA was extracted from release site soil (200 RSM2004 g^{-1}), and from this soil that had been diluted 10-fold by mixing with control, uninoculated soil. Similarly, a 10-fold dilution series to obtain CT0370 cell densities of 10^3, 10^2 and 10^1 cfu g^{-1} soil was made by mixing soil samples from the CT0370 release site with soil taken before the release (stored at 4 °C).

PCR amplification

PCR was performed in a total reaction volume of 20 μl under a layer of mineral oil (light white; Sigma) using an automated DNA thermal cycler (TRIO-Thermoblock, Biometra). RSM2004 or CT0370 DNA, prepared according to Hirsch and Skinner (1992), was used as a positive control in PCR assays. Negative controls consisted of reagent but no DNA, and of DNA extacted from uninoculated soil. For PCR amplification of soil-extracted DNA, 1–5 μl, containing 25–200 ng soil DNA representing 1–10 mg soil, was usually used as template.

PCR products were analysed by electrophoresis in horizontal agarose gels (3% NuSieve®GTG®, Flowgen, Litchfield, UK) in TBE buffer (Sambrook *et al.* 1989) followed by ethidium bromide (EtBr) staining and u.v. illumination (312 nm). The gels were photographed with Polaroid Type 665 instant film.

RSM2004 detection

Tn*5*-specific forward (primer G, bp 191–210: 5′-TCT CAT GCT GGA GTT CTT CG – 3′) and reverse (primer A, bp 613–632: 5′-ACC AGG TCA ACA GGC GGT AA – 3′) primers amplified a 442 bp (bases 191–632, Mazodier *et al.*

1985) internal region of the Tn*5* sequence that contains the bleomycin resistance gene. An *Xho*I restriction site within the fragment produces two fragments of 100 and 345 bp, which can be used to confirm the identity of the product (Fig. 1a).

PCR conditions using primers A/G were optimized using RSM2004 total genomic DNA by altering the following parameters: denaturation temperature (94 °C and 95 °C), annealing temperature (55 °C to 65 °C) and time (1 min to 2 min), primer concentration (0·1–0·6 μmol l^{-1}), Mg^{2+} ion concentration (1·5–5·5 mmol l^{-1}), reaction volumes (20, 40 and 60 μl), and the number of cycles (25–35). Three polymerases were compared: Taq DNA Polymerase (Boehringer Mannheim GmbH, Mannheim, Germany); AmpliTaq® DNA Polymerase (PE Applied Biosystems, UK); and Dynazyme (Flowgen). The Boehringer Mannheim Taq gave more product, without primer dimer artifacts, and was titrated from 0·25 to 2·5 U; the optimum concentration was 0·4 U in 20 μl. The addition of 1·25% deionized formamide and 250 μg ml^{-1} bovine serum albumin (BSA, Sigma) to the reaction mix increased the amount of product. The optimal reaction mix contained 10 mmol l^{-1} Tris-HCl pH 8·3, 50 mmol l^{-1} KCl, 3·5 mmol l^{-1} $MgCl_2$, 250 μg ml^{-1} BSA, 1·25% deionized formamide, 0·2 mmol l^{-1} dNTPS, 0·3 μmol

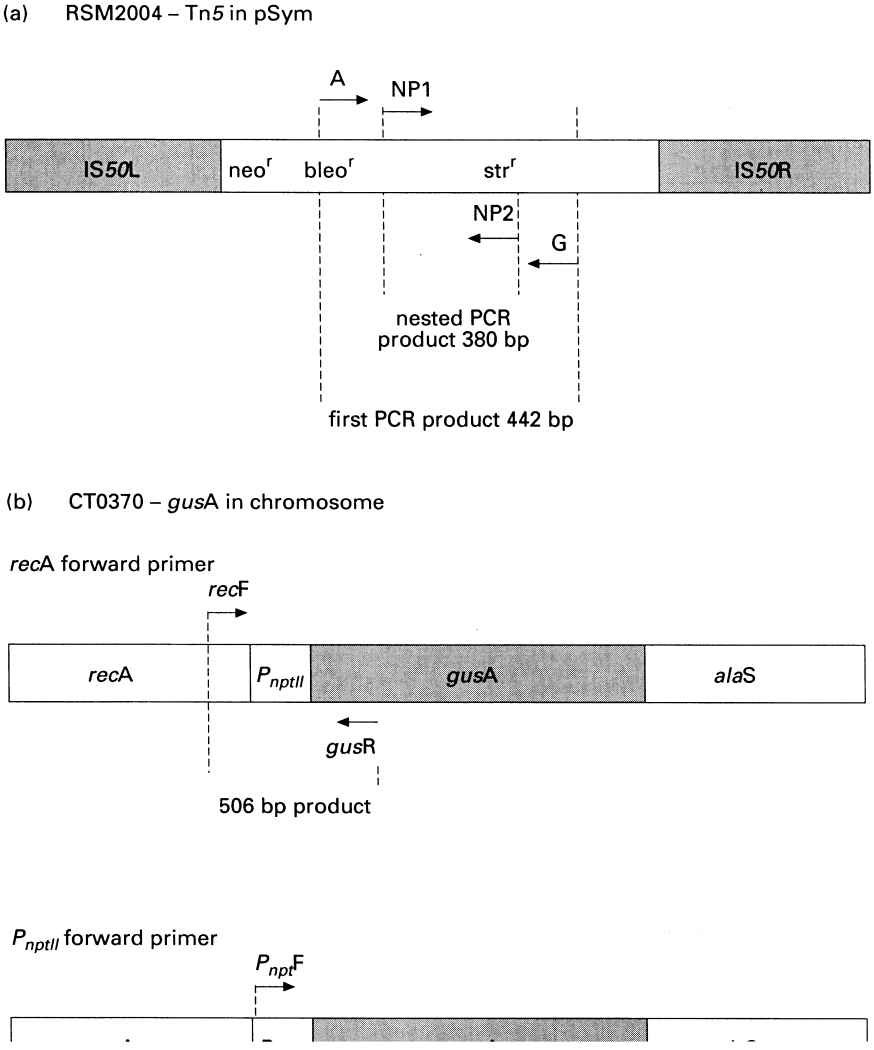

Fig. 1 Primers for PCR

l^{-1} each primer (A and G), and 0·4 U Taq Polymerase in 20 μl volume. The optimal cycling conditions were: initial denaturation at 95 °C for 2 min followed by 32 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 2 min, extension at 72 °C for 1·5 min, and a final extension at 72 °C for 5 min. The sensitivity of detection was tested by the addition of serial dilutions of RSM2004 template DNA (1 ng to 10 fg) to the PCR reaction mix.

Nested PCR was used to increase the sensitivity of detection and served as an alternative to Southern blot analysis for the confirmation of PCR products. Downstream primer NP1: 5′-AGT TGG TTC AGC TGC TGC – 3′ and upstream primer NP2: 5′-GTA ACC GGC CTC TTC ATC – 3′ (base positions 237–254 and 599–616, respectively; Mazodier *et al.* 1985) amplify a 380 bp product internal to the first 442 bp product (Fig. 1a). Conditions for nested PCR were investigated; a 2·5 μl aliquot of the primary reaction was the optimum volume to produce the 380 bp nested product in a reaction mix containing 10 mmol l^{-1} Tris-HCl pH 8·3, 50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} dNTPs, 0·3 μmol l^{-1} each primer (NP1/NP2) and 0·4 U Taq Polymerase, 20 μl final volume. Cycling conditions were: initial denaturation at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min.

CT0370 detection

Primers specific for the GUS gene insertion in CT0370 were designed (Fig. 1b); two forward primers, the first internal to the *rec*A gene (*rec*F: 5′-CGA TGG CGA TGC CAC TGC-3′) and the second in the P_{npt}II *sequence (*P_{npt}F: 5′-GCA AGC ACT CAG GGC GCA-3′) were matched with a reverse primer internal to the GUS gene (*gus*R: 5′-CCT AGC GCT TTT GAC ACC-3′). Primers *rec*F/*gus*R generated a PCR product of 506 bp, whereas P_{npt}F/*gus*R primers gave a 432 bp product.

PCR conditions were optimized for the primer sets of *rec*F/*gus*R and P_{nptII}F/*gus*R by alteration of the annealing temperature (54 °C to 61 °C) and time (1 min to 2 min). The reaction mix was as described for primers A/G but with 0·2 μ mmol l^{-1} each primer. Cycling conditions were: initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min (*rec*F/*gus*R) or 57 °C for 1·5 min (P_{nptII}F/*gus*R), extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min.

DNA probes for hybridization

To detect and verify the identities of Tn*5* PCR products, either pXS5 DNA, or the 442 bp PCR product of primers A/G were used as probes. A 1·8 kb *Bam*HI fragment from pSM26 containing the GUS gene used in the construction of CT0370 (Selbitschka *et al.* 1995) was used to detect PCR products from CT0370. Probes were labelled by random priming with DIG-11-dUPT using the Boehringer Mannheim DIG labelling kit (DIG System User's Guide, Boehringer Mannheim). The amplified DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) by Southern blotting for 48–72 h, hybridized under high stringency with the appropriate DIG-labelled DNA probe, and detected by chemiluminescence with CSPD (Boehringer Mannheim) according to the manufacturer's instructions.

Density gradient fractionation of DNA

Buoyant density centrifugation of DNA in CsCl in the presence of the intercalating dye bisbenzimide was used to separate soil DNA according to G + C content. PVPP spin-column purified DNA extracted from 9 g soil (total yield approximately 70 μg DNA) was added to CsCl solution in TE (pH 8·0, Sambrook *et al.* 1989) containing 40 μg ml^{-1} bisbenzimide (Hoechst 33258, Sigma, UK) and 3 μg RSM2001 DNA as a density marker, to a final density of 1·7 g ml^{-1}. A control containing only the RSM2001 marker DNA was also prepared. After centrifugation at 40 000 *g*, 18 °C for 48 h in 50 ml polycarbonate tubes, three distinct DNA bands were present in the gradient that contained soil DNA; the *Rhizobium* marker DNA was not clearly visible although by comparison with the control tube, it appeared to have banded between two closely spaced bands (Fig. 2). These three regions of the gradient were fractionated; bisbenzimidide was removed by three extractions with isopropanol, CsCl was removed by dialysis against 3 × TE, 1 × TNE (Sambrook *et al.* 1989), and DNA concentrated by ethanol precipitation and resuspended in 20 μl TE. A range of DNA concentrations (200, 300, 400 and 600 ng) from each of the three samples was subsequently used in PCR with Tn*5* specific primers and analysed by gel electrophoresis.

RESULTS

Optimization of PCR

PCR conditions were optimized using purified total DNA of strains RSM2004 and CT0370. Using primers A/G and bacterial DNA from pure culture, a signal was routinely obtained from Tn*5* corresponding to one to two cells of RSM2004 (i.e. 10 fg or 10^{-14} g DNA) per 20 μl reaction. Similarly, it was possible to detect specific product from the GUS gene in 10 fg strain CT0370 DNA using either primer set. These PCR products could be visualized directly on gels, and confirmed by hybridization to homologous DNA probes.

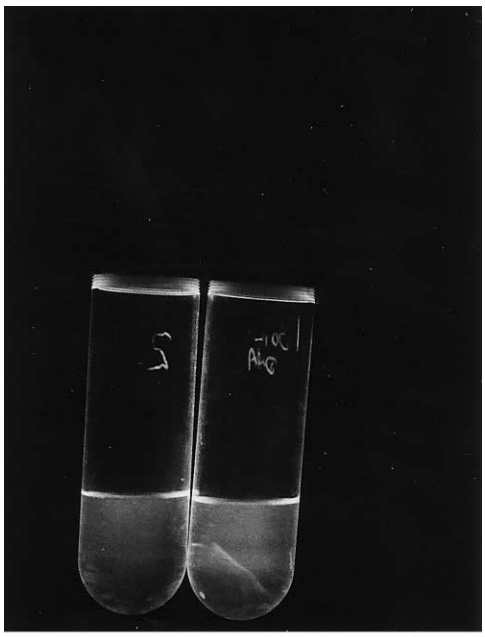

Fig. 2 Fractionation of soil-extracted DNA by density gradient centrifugation. (a), 3 μg RSM2001 DNA; (b), DNA from 9 g soil + 3 μg RSM2001 DNA

Limits of detection for Tn5-marked *R. leguminosarum* RSM2004

Plate counts indicated that 200 cfu RSM2004 g^{-1} soil survived in the field soil samples used to extract DNA. The product of 442 bp was visible but faint in agarose gels after first round PCR (primers A and G) with the equivalent of 2, 1 and 0·5 culturable cells (50, 25, 12·5 ng DNA, corresponding to the RSM2004 numbers in 12, 6, 3 mg soil, respectively). Stronger bands of 380 bp were visualized in nested PCR (primers NP1/NP2), the signal intensity correlating with the increasing concentration of target DNA per reaction (Fig. 3). Further dilution of the soil DNA gave a signal from samples containing an estimated 0·25 culturable cells (1·5 mg soil) but no signal was obtained in subsequent 10- and 100-fold dilutions. Similarly, no signals were found in DNA from control, uninoculated soil.

Strong signals of specific product after nested PCR were obtained from non-diluted soil with the equivalent of 0·3 culturable cells (25 ng DNA; 1·5 mg soil) per reaction; a faint signal was obtained from the diluted soil with the equivalent of 0·15 culturable cells (25 ng DNA; 7 mg soil) per reaction. Although this is equivalent to a detection limit of about 20 cfu g^{-1} soil, it is difficult to interpret a signal from what is theoretically less than one target sequence. Detection limits of RSM2004 in undiluted field soil were 10-fold higher, constrained by the amount of soil-extracted DNA that can be used in PCR assays.

Limits of detection for GUS-marked *R. leguminosarum* CT0370

Plate counts indicated that CT0370 was present at 10^4 cfu g^{-1} soil in the field soil used for DNA extraction, and this DNA was successfully amplified using both the *rec*F/*gus*R and P_{npt}F/*gus*R primer sets. In addition to the expected 506 bp and 432 bp products, there were many additional non-specific bands (up to 20) using soil from both the release site and control (pre-release) soil samples (Figs 4 and 5). This indicates the presence of sequences homologous to the primer sets in the indigenous soil microbial population. There were two non-specific bands co-migrating with the 506 bp product using primers *rec*F/*gus*R (Fig. 4). However, the CT0370-specific product could be detected only in soils containing CT0370; this was confirmed by its homology to a Tn*5* probe (the *nptII* promoter sequence P_{nptII} derived from Tn*5* is present within the amplified region). The primer set P_{npt}F/*gus*R avoided the need to blot and hybridize gels to detect the CT0370 signal; no non-specific bands co-migrated with the 432 kb product.

As CT0370 survived at relatively high numbers, soil-extracted DNA was serially diluted to set the PCR detection limit using primer set P_{npt}F/*gus*R. Specific product of 432 bp could still be visualized in agarose gels with approximately13 cells (1·8 ng DNA; 0·4 mg soil) per reaction but was not detectable after a further 10-fold dilution of template (Fig. 5). When release site soil was diluted with control soil, the detection limit for strain CT0370 in the extracted DNA was approximately 300 cells g^{-1} soil, i.e. 1·5 cells (50 ng DNA; 4·8 mg soil) per PCR reaction. No signal was detected with the equivalent of 0·8 cells (25 ng DNA; 2·4 mg soil) per reaction.

It is interesting to note that the background population which generated the multiple signals was relatively stable in the field; identical band patterns were seen with DNA extracted from the release site 8 months later and in other control (pre-release) samples, although the CT0370-specific bands were absent.

Fractionation of soil microbial DNA to increase detection sensitivity

The limit of detection for RSM2004 was constrained by the amount of DNA that could be used in one PCR assay; the yield of pure DNA from 1 g field soil was around 10 μg but only a fraction of this could be used in each reaction. Specific Tn*5*- and GUS products could be detected with some soil-extracted DNA samples at 300 ng and 200 ng DNA per reaction, but not with other independent samples at the same

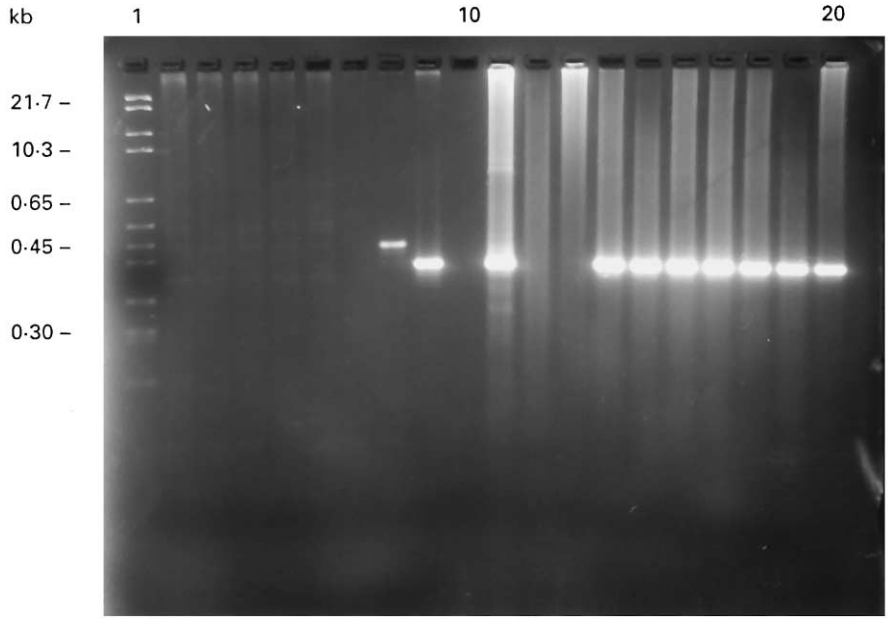

Fig. 3 Detection limit of RSM2004 in field soil by nested PCR. Lanes: 1, molecular size marker pBR328 *Bgl*I/*Hin*fI-digest (Boehringer Mannheim); 2, 50 ng soil DNA (2 cells, 12 mg soil); 3, 4, 25 ng soil DNA (1 cell, 6 mg soil); 5,6, 12·5 ng soil DNA (0·5 cells, 3 mg soil); 7, no DNA negative-control; 8, 1 pg RSM2004 DNA positive-control; 9, 20 ng RSM2004 DNA positive-control; 10, no DNA negative-control; Lanes 11–20 contain 2·5 μl from the first PCR reactions: 11, 1 pg RSM2004 DNA; 12, no DNA negative-control; 13, 14, 50 ng soil DNA; 15–17, 25 ng soil DNA; 18–20, 12·5 ng soil DNA. Products in lanes 2–8 and 9–20 were amplified using primers A/G and NP1/NP2 (nested PCR), respectively. Each lane (excluding 1) represented the total (20 μl) volume of PCR amplified mix. The reason for the smear of high molecular weight product in lane 13 is not known

concentrations. However, signals were observed with these DNA samples when they were added to the PCR mix at lower concentrations (i.e. 100, 50 or 25 ng DNA per reaction). This discrepancy between samples might be due to PCR-inhibitory contaminants from soil, co-extracted with the DNA and not entirely removed during the purification process. It could also be due to differences in the ratio of target DNA to non-target DNA as a consequence of variation in the lysis and DNA recovery efficiencies. Even if all the impurities are removed from soil DNA extracts, the threshold of detection is dependent on the ratio of target to non-target DNA; the addition of high DNA concentrations to the reaction also inhibits the PCR (Bruce *et al.* 1992; Picard *et al.* 1992). This highlights the importance of including a range of soil-extracted DNA concentrations in the PCR to avoid false negative results.

In an attempt to overcome the inhibition of specific PCR by heterogenous DNA from the background soil population, we fractionated DNA on CsCl gradients according to its G + C content, which determines the buoyant density of the DNA. Different bacterial genera differ in their nucleotide base composition, which can vary from 30% to 70% G + C; the higher the proportion of G + C residues, the denser the DNA. *Rhizobium* spp. typically have a G + C content of 59–64% (Hirsch and Skinner 1992) and thus can be enriched from total soil DNA.

A soil DNA extract in which no Tn*5* signal was detected in PCR reactions containing 200 ng soil DNA was fractionated. When 200 and 300 ng aliquots of DNA recovered from the band containing *Rhizobium* density marker were subjected to PCR, specific product was detected after gel blot hybridization with a Tn*5* probe. No Tn*5* signal was detected in fractions with higher and lower G + C contents. This was not due to removal of contaminants during CsCl centrifugation; purification of the DNA by CsCl–EtBr centrifugation, which does not separate DNA according to G + C content, did not give any Tn*5* signal.

DISCUSSION

Our Tn*5* primers gave no signal with uninoculated soil, indicating that if the soil contained indigenous DNA sequences with primer homology, they were below the limit of detection. In contrast, the signals obtained with the GUS primers indicated a substantial soil population with some DNA homology. We demonstrated that formamide and BSA increased the yield of our PCR products and the sensitivity of detection, in contrast to increasing the number of cycles which did not improve product yield (it reduced the signal but did generate non-specific products, smearing, and primer–dimer artifacts). BSA is reported to bind and stabilize single-stranded DNA to facilitate primer annealing (Romanowski *et al.* 1992) and to overcome inhibition of PCR by binding to the inhibitory components in soil (Kreader 1996; McGregor *et al.* 1996),

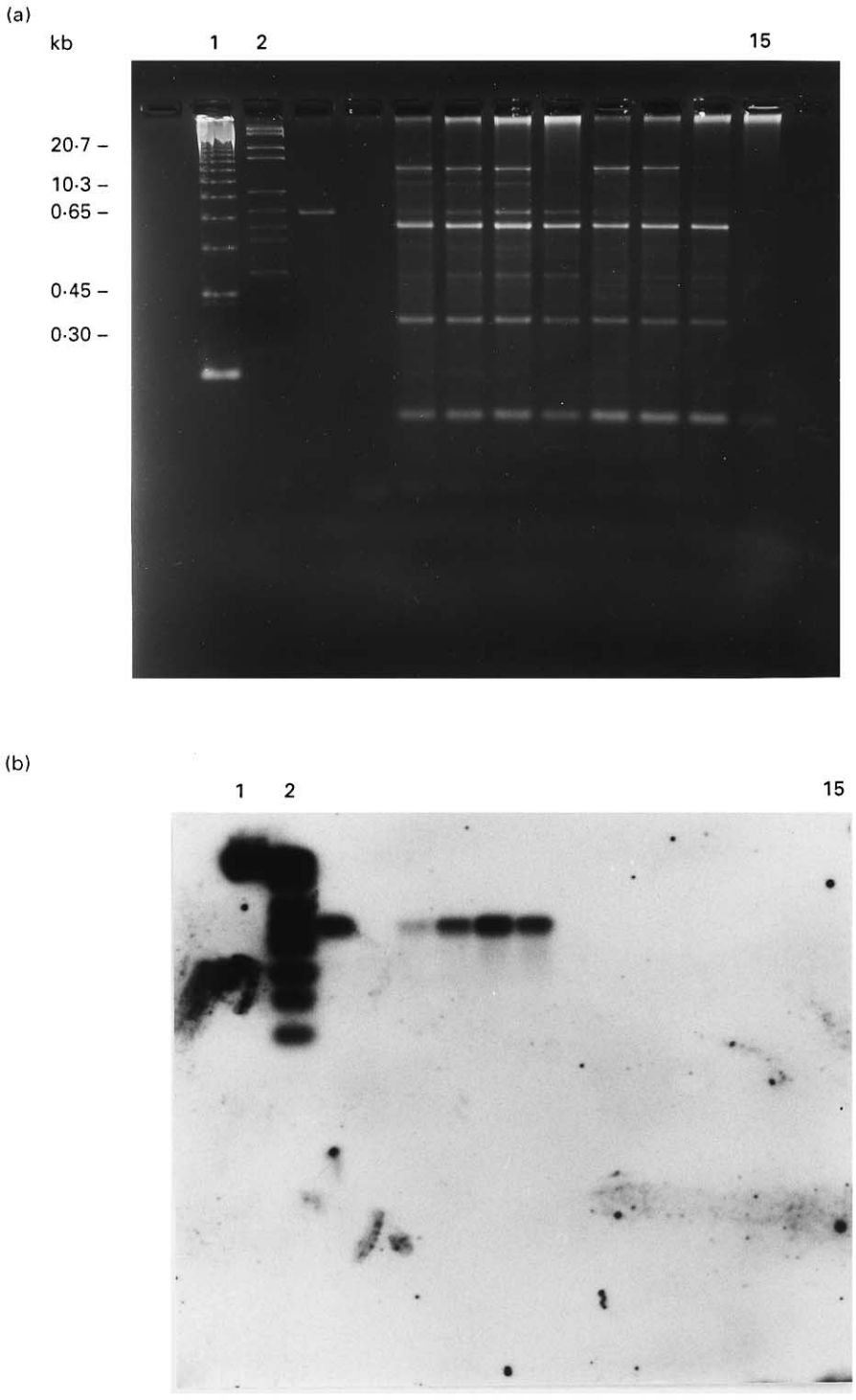

Fig. 4 Detection limits of CT0370 in field soil by PCR with primers *Rec*F/*gus*R. (a), Ethidium bromide-stained gel (3%); (b), gel blot hybridized to a Tn*5*-specific probe. Lanes: 1, 2, molecular size markers – 123 bp ladder (Gibco BRL Life Technologies), pBR328 *Bgl*I/*Hin*fI digest (Boehringer Mannheim), respectively; 3, 10 pg CT0370 DNA positive-control; 4, no DNA negative-control; 5–8, post-release soil extracted DNA at 50, 100, 200 and 300 ng, respectively; 9–12, pre-release soil extracted DNA at 50, 100, 200 and 300 ng DNA, respectively. Each lane (excluding 1 and 2) represented the total (20 μl) volume of PCR amplified mix

thereby preventing their interaction with target DNA and Taq polymerase. Formamide enhances specific primer annealing (Sarkar *et al.* 1990; Bruce *et al.* 1992). Our PCR detection of fewer than 20 culturable cells per gram of soil (one cell or less per PCR reaction) for Tn*5*-marked Rhizobia and 300 cells per gram of soil (1·5 cells per PCR reaction) for GUS-marked Rhizobia established in field soils are 10- to 1000-fold more sensitive than those reported previously for

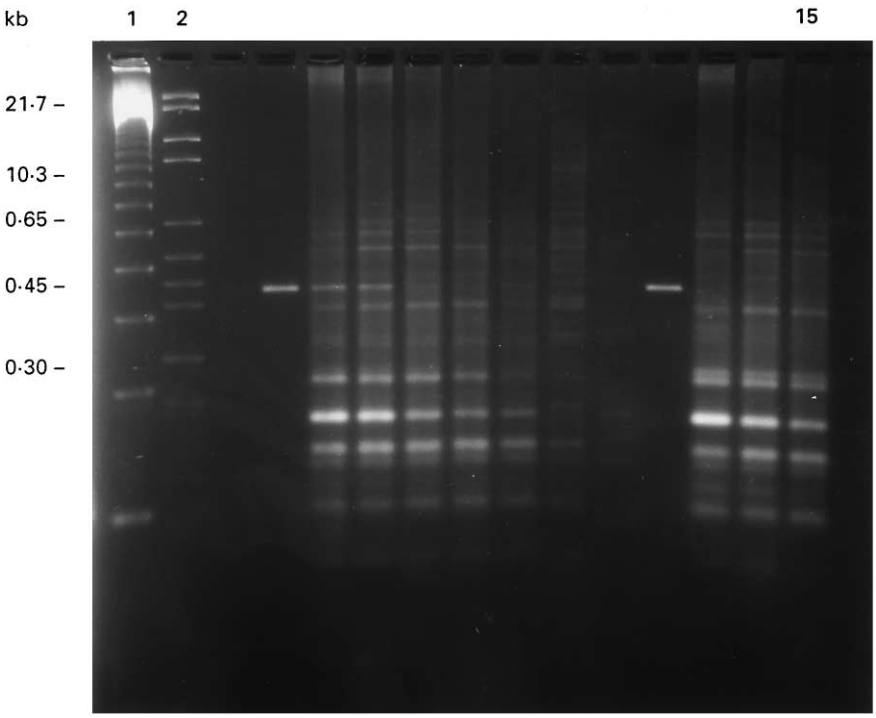

Fig 5 Detection limits for CT0370 in field soil by PCR with primers $P_{npt}F$/*gus*R. Lanes: 1, 2, molecular size markers – 123 bp ladder (Gibco BRL Life Technologies), pBR328 *Bgl*I/*Hin*fI digest (Boehringer Mannheim), respectively; 3, no DNA negative-control; 4, 10 pg CT0370 DNA positive-control; 5–11, decreasing amounts of DNA from CT0370 release site: 5, 86 ng DNA (646 cells, 20 mg soil); 6, 43 ng DNA (323 cells, 10 mg soil); 7, 22 ng DNA (161 cells, 5 mg soil); 8, 7 ng DNA (54 cells, 1·7 mg soil); 9, 1·8 ng DNA (13 cells, 0·4 mg soil); 10, 180 pg DNA (1·3 cells, 42 μg soil); 11, 18 pg DNA (0·13 cells, 4·2 μg soil); 12, 1 pg CT0370 DNA positive-control; 13–15, decreasing amounts of DNA from pre-release soil (13, 86 ng; 14, 43 ng; 15, 21 ng). Each lane (excluding 1 and 2) represented the total (20 μl) volume of PCR amplified mix

other organisms added to soil or sediments before extraction (Picard *et al.* 1992; Erb and Wagner-Döbler 1993; Herrick *et al.* 1993; Smalla *et al.* 1993; Tebbe and Vahjen 1993).

Smalla *et al.* (1993) were able to use PCR to detect target DNA from soil microcosms inoculated with 10^3 *Pseudomonas fluorescens* cfu g^{-1} dry soil even when the organism was no longer detectable on selective agar plates. *Agrobacterium tumefaciens* cells have been detected by biphasic PCR after direct DNA extraction from soil when inocula ranged from 10^7 to 10^3 cells per 100 mg soil samples, but DNA samples needed to be diluted at least 100-fold for successful amplification (Picard *et al.* 1992). A detection limit of five copies of target gene per PCR reaction was reported in sterile sediment inoculated with 100 *Pseudomonas* cells per gram (Erb and Wagner-Döbler 1993). A multi-copy marker gene in the yeast *Hansenula polymorpha*, inoculated into soil, could be detected by PCR at a limit of 10 cells per gram of soil (8 μg soil DNA; 0·5 g soil per reaction), corresponding to 80 copies of the sequence (Tebbe and Vahjen 1993).

Most investigators use hybridization of gel blots to labelled DNA probes to increase the sensitivity of detection and confirm the identity of PCR products (Erb and Wagner-Döbler 1993; Herrick *et al.* 1993; Tebbe and Vahjen 1993). Nested PCR is a sensitive alternative; it also requires probes (i.e. primers) to bind to a specific sequence internal to the PCR product, but it is much quicker. The use of nested PCR to increase the detection sensitivity and confirm the identity of Tn*5* products allowed us to screen large numbers of soil samples and obtain unambiguous results within 2 days. A single target sequence, i.e. the DNA from one bacterial cell, per reaction, gave a visible signal on a gel. In contrast, blotting, hybridization and detection to confirm Tn*5* products took more than 3 days. However, the design of primers is critical to the efficacy of PCR-based monitoring and difficulties may arise for genes with substantial homology in indigenous populations, such as the GUS marker of CT0370.

The heterogeneous DNA from the native microbial population in field soils limits sensitivity of detection using PCR. This can be overcome to a certain extent by fractionating the DNA, a promising approach which needs further development. Our results show that the sensitivity of detection can be increased significantly by DNA fractionation. The lack of Tn*5* signal in the fractions with G + C contents higher or lower than rhizobial G + C offers no evidence for transfer of the Tn*5* marker to distantly related bacterial genera with distinctly different G + C contents.

There are also practical considerations which limit detection, including the PCR reaction volume (the amount of primer and Taq polymerase), the capacity of the reaction tubes, and of the wells on the gels. We have found that the realistic detection level with nested PCR is about 50 viable cells per gram of soil (this is equivalent to PCR reactions containing one target sequence). Although this level of detection can be achieved with conventional plating for the GM rhizobial strains released at Rothamsted, it allows the possibility of sensitive monitoring of bacteria without antibiotic

resistance genes in the field, and represents an important breakthrough.

No strong evidence for significant amounts of extracellular DNA in soil has been obtained; the viable counts of the GM bacteria are not inconsistent with the PCR signals, given the errors involved in plate counts of soil dilutions and reduced plating efficiency on selective agar. Thus, signals from diluted samples estimated to contain less than 1 but more than 0·1 culturable cell are within the predicted range. However, the possibility of extracellular DNA, non-viable, or moribund cells contributing to the total amount of target DNA cannot be excluded.

The success and sensitivity of the PCR in the detection of marker DNA sequences in environmental samples depends on several factors, including DNA extraction (lysis) efficiency, ratio of target sequences to non-target sequences, purity and fragment size of extracted DNA, specificity of chosen primer set and the optimization of the PCR conditions. Our results indicate that, prior to future GM bacterial releases which require monitoring, it would be expedient to design specific primers and assess them in DNA extracted from the potential release sites, to ensure that sensitive PCR-based monitoring is feasible.

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